Ca^{2+} influx and activation of a cation current are coupled to intracellular Ca^{2+} release in peptidergic neurons of *Aplysia californica*

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1. Stimulation of inputs to bag cell neurons in the abdominal ganglion of *Aplysia californica* causes an increase in their intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). We have used thapsigargin, a specific inhibitor of the endoplasmic reticulum Ca^{2+} pump, to analyse the effects of Ca^{2+} released from intracellular stores on the electrophysiological responses of bag cell neurons.

2. Using digital imaging of fura-2-loaded isolated bag cell neurons we found that thapsigargin rapidly evoked an increase in $[Ca^{2+}]_i$ in somata, with smaller increases in neurites. Thapsigargin-induced elevation of $[Ca^{2+}]_i$ peaked at about 1 μM within 5–10 min and then decayed to basal levels by 30 min.

3. Placement of an extracellular vibrating Ca^{2+}-selective microelectrode to within 1 μm of somata revealed a relatively large steady-state Ca^{2+} efflux. Thapsigargin produced a rapid increase in Ca^{2+} influx. Changes in Ca^{2+} flux were not detected at neurites.

4. Thapsigargin produced a small depolarization in isolated bag cell neurons in artificial sea water (ASW). Sometimes enhanced depolarizations were observed when extracellular Na^+ was replaced by TEA or Tris, but not *N*-methyl-D-glucamine (NMDG). The depolarization was not blocked by 100 μM tetrodotoxin (TTX), removal of extracellular Ca^{2+} (0·5 mM EGTA) or addition of 10 mM Co^{2+} to the bath solution.

5. In voltage-clamp experiments, thapsigargin induced an inward current (I_{Tg}) that was recorded in Ca^{2+}-free media containing TEA or Tris substituted for Na^+. The apparent reversal potential of I_{Tg} was $-16{\cdot}8 \pm 1{\cdot}2$ mV in TEA-ASW. Induction of I_{Tg} was inhibited in neurons that were microinjected with the Ca^{2+} chelator BAPTA–$Dextran_{70}$ or treated with the membrane-permeant analogue BAPTA AM. Activation of I_{Tg} was not observed when Na^+ was replaced with NMDG. Manipulation of $[Na^+]_o$ and $[K^+]_o$ produced shifts in the reversal potential of I_{Tg} consistent with the underlying channels being permeable to both Na^+ and K^+.

6. Thapsigargin did not alter the amplitude or kinetics of voltage-activated Ba^{2+} currents, but in some experiments it did increase the amplitude of a component of outward K^+ current.

7. Thapsigargin neither induced bag cell neurons within the intact ganglion to depolarize and fire spontaneously, nor did it alter the frequency or duration of firing of an electrically stimulated bag cell after-discharge.

8. We conclude that thapsigargin-sensitive Ca^{2+} pools are present predominantly in the somata of bag cell neurons. Ca^{2+} that is released from thapsigargin-sensitive Ca^{2+} stores activates a non-selective cation current that may help sustain depolarization of the somata, but does not by itself trigger an after-discharge.

Many important cellular events in excitable cells are regulated by carefully controlled changes in their cytosolic free-Ca^{2+} concentration ($[Ca^{2+}]_i$). For example, in neurons, a change in the $[Ca^{2+}]_i$ alters excitability, secretion of neurotransmitters and kinetic and morphological properties of growth cones (Connor, 1986; Carafoli, 1987; Tsien & Tsien, 1990; Zheng, Felder, Connor & Poo, 1994). The important roles that Ca^{2+} influx through voltage-gated Ca^{2+} channels play in the regulation of neuronal functions have been extensively studied (Kaczmarek & Levitan, 1987; Hess, 1990; Tsien & Tsien, 1990). However, Ca^{2+} can also enter neurons by other mechanisms, such as receptor-operated channels and ion exchangers (Blaustein, 1988). Once Ca^{2+} reaches the inner surface of the plasma membrane it may activate exocytosis through activating synaptic vesicle proteins (Jahn & Sudhof, 1993), gate different types of ion channels (Partridge & Swandulla, 1987; Tsien & Tsien, 1990), stimulate the release of Ca^{2+} from intracellular stores (Neering & McBurney, 1984; Lipscombe, Madison, Poenie, Reuter & Tsien, 1988; Tsien & Tsien, 1990; Friel & Tsien, 1992) or alter the activity of membrane transport proteins.

Intracellular Ca^{2+} stores have been classified on the basis of the different ligands that stimulate Ca^{2+} release. Two dominant stores in neurons are those regulated by inositol 1,4,5,-trisphosphate (IP_3) (Higashida & Brown, 1986; Payne & Fein 1987; Fink, Connor & Kaczmarek, 1988; Thayer, Perney & Miller, 1988) and caffeine (Lipscombe *et al.* 1988). Following stimulation of a neuron, several different patterns of changes in $[Ca^{2+}]_i$ have been described. These include regenerative Ca^{2+} transients, Ca^{2+} waves and Ca^{2+} oscillations. Each of these responses is shaped by the interplay between Ca^{2+} reuptake into internal stores, Ca^{2+} buffering by intracellular proteins and Ca^{2+} export across the plasma membrane (Blaustein, 1988; Tsien & Tsien, 1990). However, the precise functions of the multiple types of intracellular Ca^{2+} stores in neuronal Ca^{2+} homeostasis and the physiological role(s) of the Ca^{2+} that is discharged from these stores are not fully understood.

We have used thapsigargin, a Ca^{2+}-ATPase inhibitor that promotes Ca^{2+} release from intracellular compartments (Thastrup, Cullen, Drobak, Hanley & Dawson, 1990), to study the actions of intracellular Ca^{2+} release in the bag cell neurons of *Aplysia*. In response to brief electrical stimulation of afferents, the bag cell neurons from the abdominal ganglion of *Aplysia* fire a prolonged burst (~30 min) of action potentials known as the after-discharge (Kaczmarek, Jennings & Strumwasser, 1978). This causes the secretion of several neuroactive peptides, including egg-laying hormone from the bag cell neurons which, *in vivo*, triggers a sequence of reproductive behaviours that culminates in egg laying (Conn & Kaczmarek, 1989).

Activation of the after-discharge is associated with IP_3 production (Fink *et al.* 1988) and recent experiments (Fisher, Levy & Kaczmarek, 1994) have suggested that the after-discharge triggers Ca^{2+} release from intracellular stores. Little is known, however, about spatial and temporal properties of intracellular Ca^{2+} release in bag cell neurons. We have now used intracellular and extracellular recordings in combination with intracellular Ca^{2+} imaging to provide evidence that Ca^{2+} discharged from thapsigargin-sensitive compartments stimulates a depolarizing non-selective cation conductance. Activation of such a non-selective cation current may contribute to the maintenance and propagation of action potentials in the intact nervous system during an after-discharge.

METHODS

Isolation of bag cell neurons

Adult *Aplysia californica* (200–250 g) were anaesthetized by injection of isotonic $MgCl_2$ (50% of body weight) and the abdominal ganglia, along with the pleuroabdominal connectives, were excised. To make primary cultures of bag cell neurons, ganglia were incubated in a neutral protease solution (Dispase, 40 mg in 3 ml water) for 18 h at 19–22 °C. Bag cell clusters were then dissected from their surrounding connective tissue and the neurons were plated using a Pasteur pipette into culture dishes containing artificial sea water (ASW). For experiments in which fluorescence measurements were required, the neurons were plated on #1 glass microscope coverslips coated with poly-lysine.

Intracellular Ca^{2+} imaging with fura-2

Fura-2 (Grynkiewicz, Poenie & Tsien, 1985) was microinjected into somata (50–100 μM) by pressure ejection from intracellular microelectrodes (electrical resistance >30 MΩ when filled with 3 M KCl). Injections required 20–30 s and neurons were then allowed to equilibrate for at least 30 min. For electrical recording and stimulation, the neurons were repenetrated using microelectrodes filled with 3 M KCl. Free $[Ca^{2+}]_i$ was calculated from paired ratio images of fura-2 fluorescence obtained using 340 and 380 nm excitation. Details of this method as well as the imaging apparatus have been described previously (Connor, 1986; Fink *et al.* 1988; Knox, Quattrocki, Connor & Kaczmarek, 1992). Acquisition time for one frame pair was approximately 800 ms. Action potential-driven Ca^{2+} influx was induced by injecting intracellular current pulses (0·5–1 nA, 75 ms, 3 Hz). Correction for background fluorescence and camera-dark current was carried out as follows. Proper focus of neurites and somata was first determined visually under UV excitation and then the field of excitation (~250 μm diameter) was moved to a nearby, cell-free location and exposures of the proper duration were taken at both 340 and 380 nm excitation. These images were stored in computer RAM and subtracted from all subsequent cell images. With this correction, the mean background signal measured in cell-free areas was less than 1 arbitrary unit (a.u.) and the standard deviation of this background was ~0·5 a.u.. Minimum neurite fluorescence used in analysis was always >5 a.u. and generally well above this value (20–100 a.u.). Because of the large discrepancy in size, somata fluorescence generally exceeded 1000 a.u.. Cell data were masked such that areas where the 380 nm signal fell below 5 a.u. during the peak response did not appear in any of the ratio images. In making the final ratio images, displayed individual 340 and 380 nm images were filtered (low pass, recursive, with nearest neighbour pixels weighted by 0·25) before division, enabling better use of colour display by reducing extreme ratio pixel values during peak response. Data for tabulation were always checked for proper background correction by measuring residual signals in cell-free areas of images near the structures of interest. When filling the cells, fura-2 was injected until the autofluorescence of the soma

comprised no more than about 5% of total fluorescence with 380 nm excitation. For some experiments Ca^{2+} stores in isolated bag cell neurons were first artificially loaded with Ca^{2+} by exposure to KCl (30 mM) in normal ASW (containing (mM): 460 NaCl, 10·4 KCl, 11 $CaCl_2$, 55 $MgCl_2$ and 15 Hepes; pH 7·8; Loechner, Knox, Connor & Kaczmarek, 1992) for 2 min so as to increase the amount of Ca^{2+} within the stores. After Ca^{2+} loading, neurons were returned to Ca^{2+}-free solution (no added Ca^{2+}, 0·5 mM EGTA unless otherwise stated).

Extracellular recording of Ca^{2+} flux

The vibrating Ca^{2+} electrode was used to measure changes in extracellular steady-state Ca^{2+} gradients maintained by the plasma membrane (Smith, Sanger & Jaffe, 1994). Using video-enhanced microscopy for guidance, a microelectrode tip filled with a Ca^{2+}-sensitive ionophore (FLUKA Ca^{2+} ionophore I-cocktail A, Roukoukoma, NY, USA) was moved to within 1 or 2 μm of the plasma membrane. Using software control (DVIS 10 software, National Vibrating Probe Facility, Marine Biological Laboratory, Woods Hole, MA, USA) of micromanipulator movement, the electrode was moved 10 μm to and from the plasma membrane at a frequency of (0·3–0·5 Hz). The slow vibration frequency is necessary so that a steady-state Ca^{2+} concentration is attained at both positions. The voltage difference (δV) between the two positions is proportional to the Ca^{2+} gradient (δC) between the two positions. Differential voltages measured at the cell are compared with background values taken approximately 40 μm from the plasma membrane. Ca^{2+} flux (J) values were computed from δV measurements from the equation, $J = -D\delta C/\delta r$, where J is Ca^{2+} flux in μmol $cm^{-2}s^{-1}$, δr is the vibrational amplitude of the electrode in cm and D is the aqueous diffusion constant for Ca^{2+} (8×10^{-6} cm^2 s^{-1}). A derivation of the equation is described in Smith *et al.* (1994). Fast changes in Ca^{2+} flux, such as through irregular channel openings, will not be recorded by this technique, in part due to their sigmoidal activation but also because of the ionophore response time (90% < 5 s; FLUKA specifications). Flux values are therefore underestimated, being influenced by both the response time and the distance from the source.

Microelectrodes were pulled from 1·5 mm glass (TW 150-4; WPI Inc., New Haven, CT, USA) using a P-97 Flaming–Brown puller (Sutter Instrument Co., Novato, CA, USA) with a tip length of ~1 mm and a tip diameter of 2–4 μm. Electrodes were silanized to stabilize the 30 μm ionophore column during recordings. Electrolyte (filtered 100 mM $CaCl_2$ in 0·5% agar gel) was front filled with the ionophore. Electrodes were inserted into conventional Ag–AgCl pellet containing electrode holders. Electrode movement in x, y and z planes and vibrational angle were set by an orthogonal array of stepper motors attached to the micromanipulator. This system allows movement in the submicron range (minimum practical value = 0·3 μm). Voltage was sampled at 1 KHz by an A/D board (DT-2800, Data Translations, Marlboro, MA, USA) with a dynamic range of ±10 V. A 1000-fold gain amplifier was inserted between the headstage and computer, which allowed low microvolt changes in δV to be resolved. During the dwell time of the electrode at the two positions, data were averaged to yield ten mean values. The three values measured immediately after movement were rejected. The remaining values were compared with the overall mean value from the preceding position. Ten separate δV values were computed and fed into a running average. Values of δV were converted to flux values off-line.

Current- and voltage-clamp measurements

Current-clamp recordings were made with microelectrodes (electrical resistance 7–12 MΩ when filled with 3 M KCl) pulled with a Sutter Instruments P-87 puller. Neurons were impaled and membrane potential recordings amplified using an Axoclamp 2A (Axon Instruments) controlled by a Compaq 386/20e host computer. Signals were acquired and analysed with pCLAMP 5.0 (Axon Instruments). Action potentials were stimulated by intracellular injection of depolarizing current pulses (0·2–0·5 nA; 130–150 ms).

Single-electrode voltage-clamp (Axoclamp 2A, Axon Instruments) measurements were made with microelectrodes identical to those described above. Thapsigargin-induced currents were measured in a variety of isosmotically substituted Ca^{2+}-free media (containing 2 mM EGTA), in which Na^+ was replaced by TEA-Cl or Tris-Cl. Neurons were hyperpolarized from a holding potential of −40 or −60 mV to −90 mV and sequentially depolarized (2–3 s pulse) to +20 mV in 10 mV increments. Total current flowing at the end of each pulse was measured and used to construct I–V plots. Induced currents were obtained by subtracting current amplitudes after thapsigargin application from control currents.

Voltage-activated Ba^{2+} currents were measured in substituted ASW in which NaCl and KCl were replaced with 460 mM TEA-Cl and 10·4 mM CsCl, respectively. Neurons were depolarized to test potentials between −30 and +20 mV (300 ms pulse; interpulse interval, 5 s) in 10 mV increments from a membrane holding potential of −60 mV. Peak currents were plotted against test potential. Leak currents were subtracted from ionic currents on line using a $P/4$ pulse protocol. Data were digitized, stored and analysed using pCLAMP 5.0 (Axon Instruments). Voltage-activated K^+ currents were recorded from neurons in Ca^{2+}-free (containing 2 mM EGTA) Tris-substituted ASW, in which Na^+ was replaced by Tris.

Drug application

All current- and voltage-clamp recordings were carried out in 35 mm tissue culture dishes containing 2 or 3 ml of media. Thapsigargin (L. C. labs, Woburn, MA, USA) was bath applied to final concentrations ranging from 0·5 to 7·0 μM (highest [DMSO], 0·04%), which in control experiments had no effect on membrane potential or ionic currents. In some after-discharge experiments the higher thapsigargin concentration of 50 μM was used. BAPTA–$Dextran_{70}$ (BAPTA-free acid conjugated to Dextran (MW, 70 000); Molecular Probes), which is excluded from intracellular membranous compartments, was injected into somata by pressure ejection (Picospritzer II, General Valve Corporation, East Hanover, NJ, USA) from intracellular microelectrodes (electrical resistance 30–50 MΩ when filled with 3 M KCl). Microelectrode tips were filled with a stock solution of BAPTA–$Dextran_{70}$ (0·01 mg ml^{-1} deionized water). A small amount of 0·02% Lucifer Yellow was mixed with BAPTA–$Dextran_{70}$ so that successful microinjections could be determined from visualization of intracellular fluorescence. For a 50 μm diameter bag cell neuron, the estimated intracellular [BAPTA] is ~800 nM. Assuming a simple bimolecular interaction between Ca^{2+} and BAPTA (K_d, 450 nM), this amount of BAPTA would lower the resting $[Ca^{2+}]_i$ to ~80 nM and would attenuate the peak $[Ca^{2+}]_i$ following thapsigargin treatment to ~340 nM.

RESULTS

Fura-2 measurements

Isolated bag cell neurons were injected with the fluorescent Ca^{2+} indicator, fura-2. Thapsigargin (2–7 μM) was bath applied and produced a rise in the $[Ca^{2+}]_i$. This occurred in both normal ASW ($n = 10$) and Ca^{2+}-free media ($n = 27$),

indicating that Ca^{2+} is released from internal stores and not from Ca^{2+} entry via plasma membrane pathways (Fig. 1*A a*). The onset of Ca^{2+} release began within a minute and reached maximum by about 3–5 min after the addition of thapsigargin. Thereafter the $[Ca^{2+}]_i$ declined towards control levels within 30 min (Fig. 1*C*).

The effect of thapsigargin on Ca^{2+} concentration that occurred in neurites was generally smaller than it was in somata. Measurement of intracellular Ca^{2+} concentrations at different locations revealed a standing Ca^{2+} gradient between somata and neurites. An example of such a Ca^{2+} gradient in a resting neuron is shown in Fig. 1*A* and *B*, where $[Ca^{2+}]_i$ in the distal neurite was 32% of the corresponding $[Ca^{2+}]_i$ measured in the somata. When thapsigargin was added it produced a smaller relative change in the $[Ca^{2+}]_i$ in the neurite. In Ca^{2+}-free ASW the mean control $[Ca^{2+}]_i$ of somata was 315 ± 10 nM ($n = 20$, mean ± S.E.M.) and the mean maximal $[Ca^{2+}]_i$ after thapsigargin treatment was 950 ± 110 nM ($n = 20$). The corresponding values for

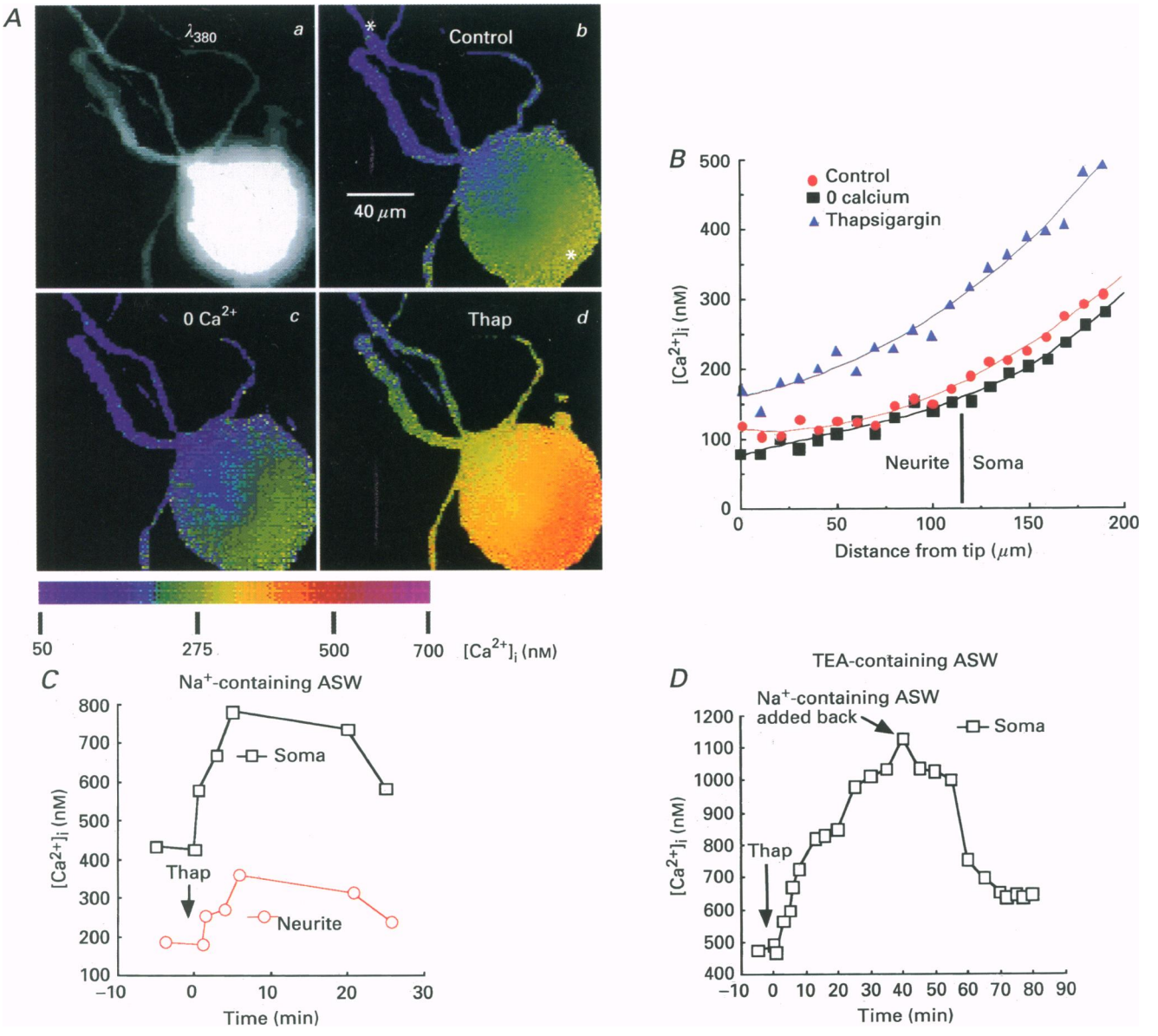

Figure 1. Fura-2 imaging of $[Ca^{2+}]_i$ in somata and neurites of bag cell neurons during thapsigargin stimulation

A, sequence of ratio images of a neuron in normal ASW (*b*), in Ca^{2+}-free media before (*c*) and 10 min after the addition of 4 μM thapsigargin (*d*). *A a* shows a raw fluorescence image of the cell evoked by 380 nm excitation. Quantitative $[Ca^{2+}]_i$ measurements were obtained from a 10 × 10 pixel box. The $[Ca^{2+}]_i$ values determined at multiple cell locations between the two asterisks in *A b* are plotted in *B*. *C* and *D* show time courses of the change in $[Ca^{2+}]_i$ in response to addition of thapsigargin in 2 neurons. The data shown in *C* were collected in normal ASW and the data in *D* were collected in Na^+-free TEA-ASW.

neurites were 175 ± 27 nM before, and 305 ± 52 nM after thapsigargin treatment, respectively.

In experiments carried out in Ca^{2+}-containing ASW, thapsigargin neither altered the time course nor the pattern of action potential-driven Ca^{2+} influx in somata or neurites (data not shown). Comparison with untreated cells showed that thapsigargin did not alter the rate at which Ca^{2+} was buffered by reuptake and/or extruded from neurons following the large Ca^{2+} load that occurs during a train of action potentials (data not shown). These results suggest that thapsigargin-sensitive Ca^{2+} stores probably contribute little to the removal of Ca^{2+} that accumulates in the cytoplasm during action potentials.

Replacement of extracellular Na^+ with TEA changed the pattern of the thapsigargin response. Under these conditions, the increase in $[Ca^{2+}]_i$ did not decline to control values as it did in Na^+-containing ASW in the continuous presence of thapsigargin, but continued to rise over 20–40 min ($n = 6$). However, as shown in Fig. 1*D*, when cells were returned to Na^+-containing ASW, $[Ca^{2+}]_i$ began to fall with a similar time course to that observed in Na^+-containing ASW. This observation suggests that Ca^{2+} is extruded from thapsigargin-stimulated neurons by a mechanism that is dependent on extracellular Na^+, possibly by a Na^+–Ca^{2+} exchanger.

Collectively the fura-2 measurements directly demonstrate that release of Ca^{2+} from internal stores occurs in somata and neurites of thapsigargin-stimulated neurons and that the Ca^{2+} that accumulates in the cytoplasm is largely removed by a mechanism that is dependent upon extracellular Na^+.

Ca^{2+} flux measurements

Figure 2*A* shows an example of a vibrating Ca^{2+}-selective microelectrode recording from an unstimulated neuron. The sketch shows the two recording positions P1 and P2 close to the cell body and a third position, P3, more distal from the cell. The recording system was configured such that negative flux values correspond to net Ca^{2+} efflux. When the microelectrode was positioned more than 40 μm away from the plasma membrane a Ca^{2+} flux signal could not be detected. Recording between P3 and P4, therefore, corresponds to the background (BG) signals as shown in Fig. 2*A* and *B*. When microelectrodes were moved to their recording position (vibrating between P1 and P2), as indicated by the large arrows in Fig. 2*A* and *B*, steady-state Ca^{2+} efflux was recorded in all eight unstimulated neurons examined.

Typically, bath applications of thapsigargin to a final concentration of 7 μM caused a rapid reduction in the steady-state measured efflux (7 of 8 cells). After 1 min no further reduction was observed as illustrated in Fig. 2*B*. After approximately 20 min, Ca^{2+} efflux steadily increased to a value close to the pre-thapsigargin level.

There are two possible explanations for the reduction of the efflux value. As the probe technique generates a non-invasive differential measurement of Ca^{2+} activity at the tip of the electrode, it only provides a net measure of Ca^{2+} flux between these points. As, in these experiments, it is in proximity to the plasma membrane, we can assume it measures net Ca^{2+} movement across the surface in its immediate vicinity. Therefore, the suppression of net Ca^{2+} efflux reported here

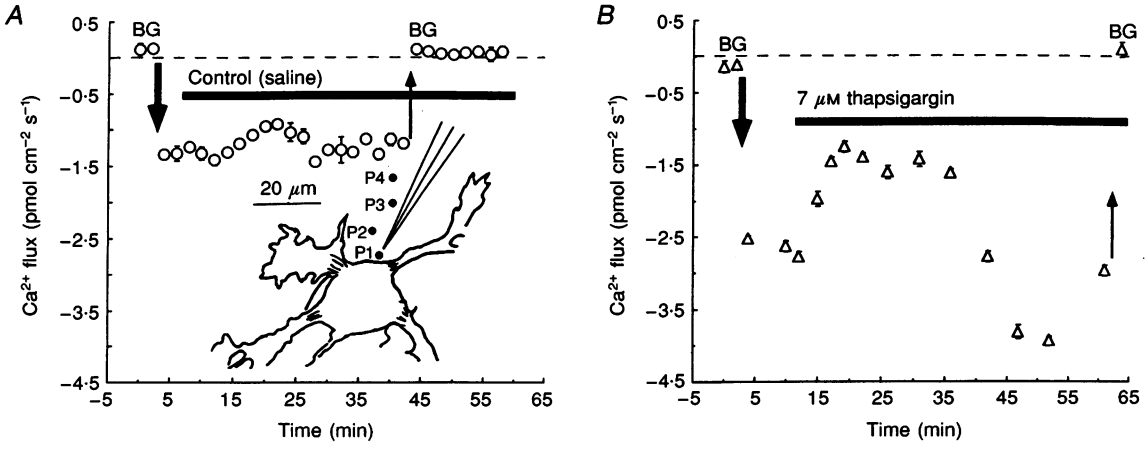

Figure 2. Measurement of membrane Ca^{2+} flux with a vibrating microelectrode

A, Ca^{2+} flux recorded from a control, unstimulated cell and a sketch of the recording configuration. P1 and P2 represent the two positions that the electrode vibrates between when recording membrane Ca^{2+} flux. The large arrows in both *A* and *B* indicate when the microelectrode was moved from background (BG) position to recording position. The smaller arrows indicate when the microelectrode was moved back from the recording position to the background position. BG signal was determined approximately 40 μm from the plasma membrane as the electrode vibrated between P3 and P4. *B*, addition of 7 μM thapsigargin produced a transient (~1 min) enhancement of Ca^{2+} efflux followed by a sustained decline in efflux (~25 min). Subsequently, after a short overshoot, Ca^{2+} flux returned close to its initial value. Similar results were obtained from 6 other neurons.

could result from changes in either the activity of the membrane transport proteins, the Ca^{2+}-ATPase and/or Na^+–Ca^{2+} exchanger, or a general enhancement of Ca^{2+} entry (Ca^{2+} influx channels) reducing the measured signal by simple subtraction. As thapsigargin is shown in this study to greatly increase free cytosolic Ca^{2+}, and as there is no evidence for a direct action of this compound on plasma membrane transport proteins, the simplest explanation for the observed response is a net increase in Ca^{2+} influx (see Discussion). The time course of this response closely matches the thapsigargin-induced rise in cytosolic free Ca^{2+}.

The second phase of the response, the steady increase in efflux back to the pretreatment levels corresponding with the return of the cytosolic free-Ca^{2+} level to control levels, may reflect the activities of either the plasma membrane Ca^{2+}-ATPase or the Na^+–Ca^{2+} exchanger.

Thapsigargin depolarizes bag cell neurons

Figure 3*A* shows the effects of thapsigargin (1 μM) on the membrane potential of five isolated bag cell neurons in different ion-substituted media. In both normal and Ca^{2+}-free ASW (Fig. 3*A a*), thapsigargin caused a slow depolarization. For each experiment described below (unless otherwise stated) the extracellular solution was Ca^{2+} free. When Na^+ was replaced with TEA or Tris (Fig. 3*A b* and *A c*), the amplitude of the depolarization was sometimes larger ($8{\cdot}6 \pm 2{\cdot}7$ mV ($n = 12$) and $7{\cdot}7 \pm 1{\cdot}3$ mV ($n = 3$), respectively) than it was in the presence of Na^+ ($4{\cdot}7 \pm 0{\cdot}82$ mV, $n = 8$). One explanation for this enhanced depolarization is that removal of extracellular Na^+ inhibits Ca^{2+} efflux which, in turn, allows a higher local $[Ca^{2+}]_i$ to accumulate at the site(s) where Ca^{2+} triggers the depolarization. The depolarization in TEA-ASW is also

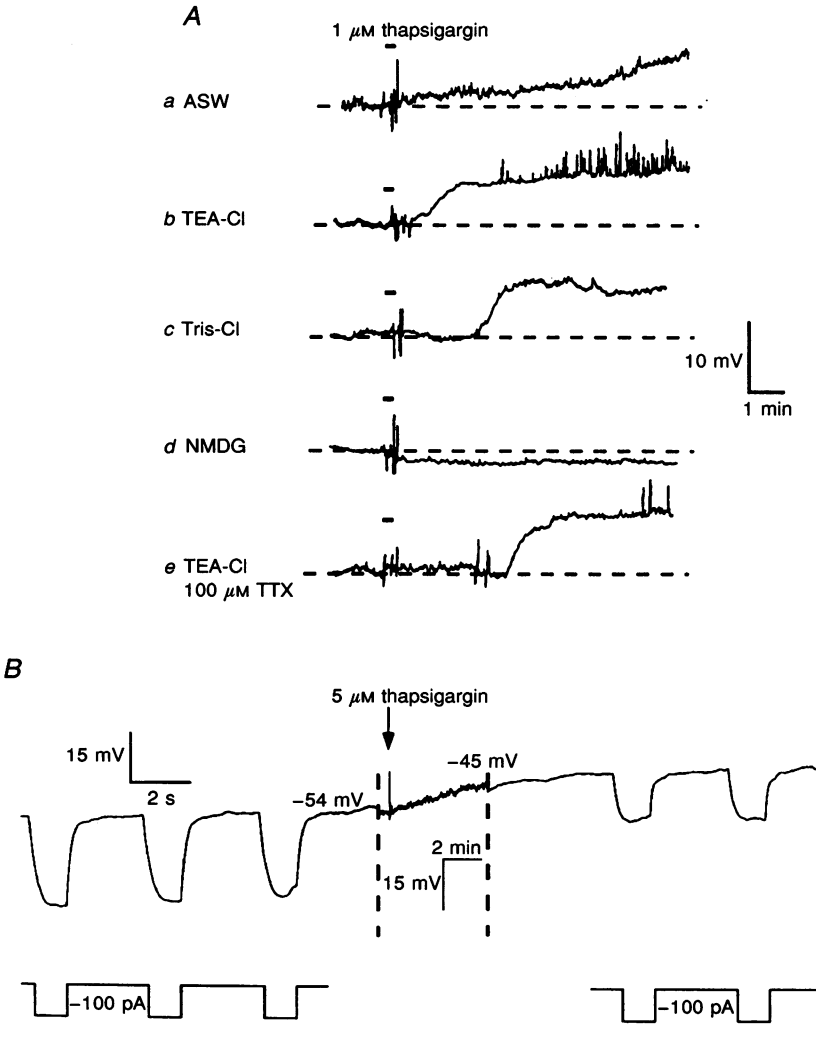

Figure 3. Thapsigargin-induced depolarization of bag cell neurons

A, records of membrane potential from 5 neurons under different pharmacological conditions. For each cell 1 μM thapsigargin was bath applied, as indicated by the bar. In *e*, 100 μM TTX was added to the bath 40 min before the addition of thapsigargin. The resting potential of each of these neurons was between −52 and −67 mV. *B*, example of the thapsigargin-induced depolarization and the associated decrease in membrane input resistance measured as the response to hyperpolarizing current pulses. The resting membrane potential of the neuron was −54 mV.

likely to be larger because of the block of Ca^{2+}-activated K^+ channels by TEA. As shown in Fig. 3*A d*, when Na^+ was replaced by *N*-methyl-D-glucamine (NMDG), addition of thapsigargin caused a 3 mV hyperpolarization, presumably by activating a Ca^{2+}-dependent K^+ conductance. In response to thapsigargin application the mean change in membrane potential of neurons in NMDG was $-2{\cdot}4 \pm 0{\cdot}23$ mV ($n = 4$). When external Na^+ was replaced with TEA-Br, thapsigargin caused depolarizations that were similar to those observed with the Cl^- salt, indicating that the depolarization did not depend upon external Cl^-.

Pretreatment of neurons with 100 μM TTX for at least 40 min (Fig. 3*A e*) or the addition of the Ca^{2+} channel blocker $CoCl_2$ (10 mM) did not prevent the thapsigargin-induced depolarization, ruling out a mechanism involving the voltage-gated Na^+ and Ca^{2+} channels in these neurons. In some experiments performed in Na^+-containing media the depolarizations reversed when neurons were perfused with thapsigargin-free ASW. Thapsigargin also produced an increase in membrane conductance measured by the amplitude of membrane potential responses to intracellularly injected hyperpolarizing current pulses. In the example shown in Fig. 3*B*, a 9 mV depolarization was associated with a 42% decrease in input resistance of the cell 5 min after the addition of thapsigargin.

Activation of a non-selective cation current (I_{Tg})

The nature of the ionic current(s) underlying the thapsigargin-induced depolarization was investigated using a single-microelectrode voltage clamp. Figure 4*A* shows steady-state currents in Ca^{2+}-free ASW, in which NaCl was replaced by TEA-Cl, flowing in an isolated neuron that was hyperpolarized to −90 mV from a holding potential of −40 mV and then depolarized to +40 mV in 10 mV step increments before and 5 min after application of 2 μM thapsigargin. The third family of traces represents the difference currents measured 5 min after the addition of thapsigargin. The *I–V* relations of the measured currents (Fig. 4*B a*) and of the induced current I_{Tg} (Fig. 4*B b*) show the characteristics of a steady-state current that is relatively linear over the voltage range −90 to +20 mV. An appreciable amount of I_{Tg} could be detected within 2 min and it usually reached a maximum amplitude (0·2–0·4 nA at −90 mV) within 5–10 min. The onset and development of I_{Tg} paralleled the general time course of the depolarizations as well as the time course of the thapsigargin-stimulated rise in $[Ca^{2+}]_i$ and changes in transmembrane Ca^{2+} flux. I_{Tg} was never observed in control experiments (bath application of 30 μl of vehicle).

The mean estimated reversal potential of I_{Tg} in Na^+-containing ASW was $-19{\cdot}2 \pm 0{\cdot}94$ mV ($n = 8$), and in

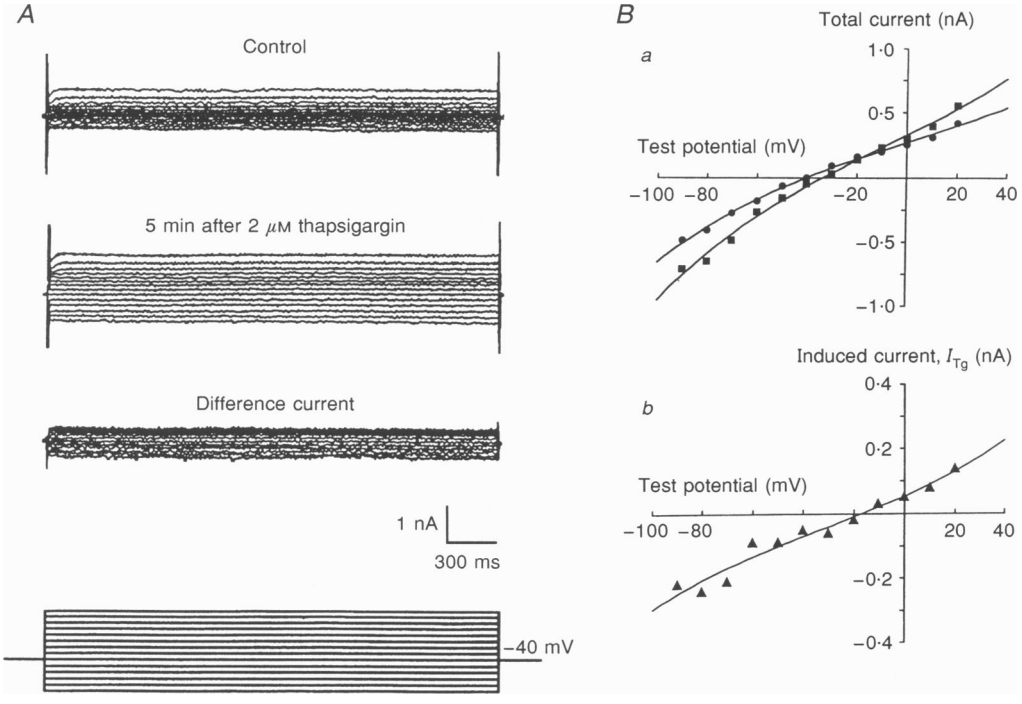

Figure 4. Activation of an inward current by thapsigargin

A, records of membrane currents at different voltages before and after the addition of thapsigargin, from a neuron bathed in TEA-ASW. Difference currents represent thapsigargin-induced currents, and were obtained by subtracting control currents from currents recorded 5 min after 2 μM thapsigargin. *Ba* and *b* shows the corresponding *I–V* relations: ●, control and ■, 5 min after 2 μM thapsigargin (*Ba*); ▲ subtracted induced current (*Bb*). The estimated reversal potential of the induced current in this example was −17·3 mV.

TEA- and Tris-ASW the estimated reversal potentials were $-16{\cdot}8 \pm 1{\cdot}2$ mV ($n = 8$) and $-18{\cdot}3 \pm 1{\cdot}4$ mV ($n = 4$), respectively. I_{Tg} was not observed when Na^+ was replaced by NMDG, which is consistent with the absence of a thapsigargin-induced depolarization in NMDG substituted media.

To test if I_{Tg} was activated directly in response to the release of Ca^{2+} from internal stores, BAPTA–Dextran$_{70}$, a cytoplasmic Ca^{2+} chelator, was microinjected into neurons prior to application of thapsigargin. The size of the high molecular weight conjugate (MW, 70 000) excludes it from intracellular membrane compartments. Figure 5*B* shows a Nomarski image (*a*) and fluorescence image (*b*) of the same field containing two neurons. The cell on the left was injected with BAPTA–Dextran$_{70}$ plus a small amount of 0·02% Lucifer Yellow, which was used to gauge successful injections. The injected cells were allowed to equilibrate for at least 10 min before starting the voltage-clamp experiments. Figure 5*A* shows currents before and 10 min after the addition of thapsigargin in a neuron injected with BAPTA–Dextran$_{70}$. In contrast to the currents shown in Fig. 4*A*, thapsigargin failed to induce I_{Tg} in the BAPTA-injected cells. Similar results were obtained in five other neurons. In control experiments, microinjection of BAPTA–Dextran$_{70}$ and Lucifer Yellow failed to induce I_{Tg}. These results, and those obtained with the membrane-permeant analogue BAPTA AM (acetoxymethyl ester form of BAPTA) ($n = 4$), strongly suggest that I_{Tg} is activated directly by Ca^{2+} discharged from intracellular stores.

Collectively, the pharmacology of the depolarization and the I_{Tg} and the fact that I_{Tg} reversed at approximately −20 mV suggest that it is a voltage-independent non-selective cation

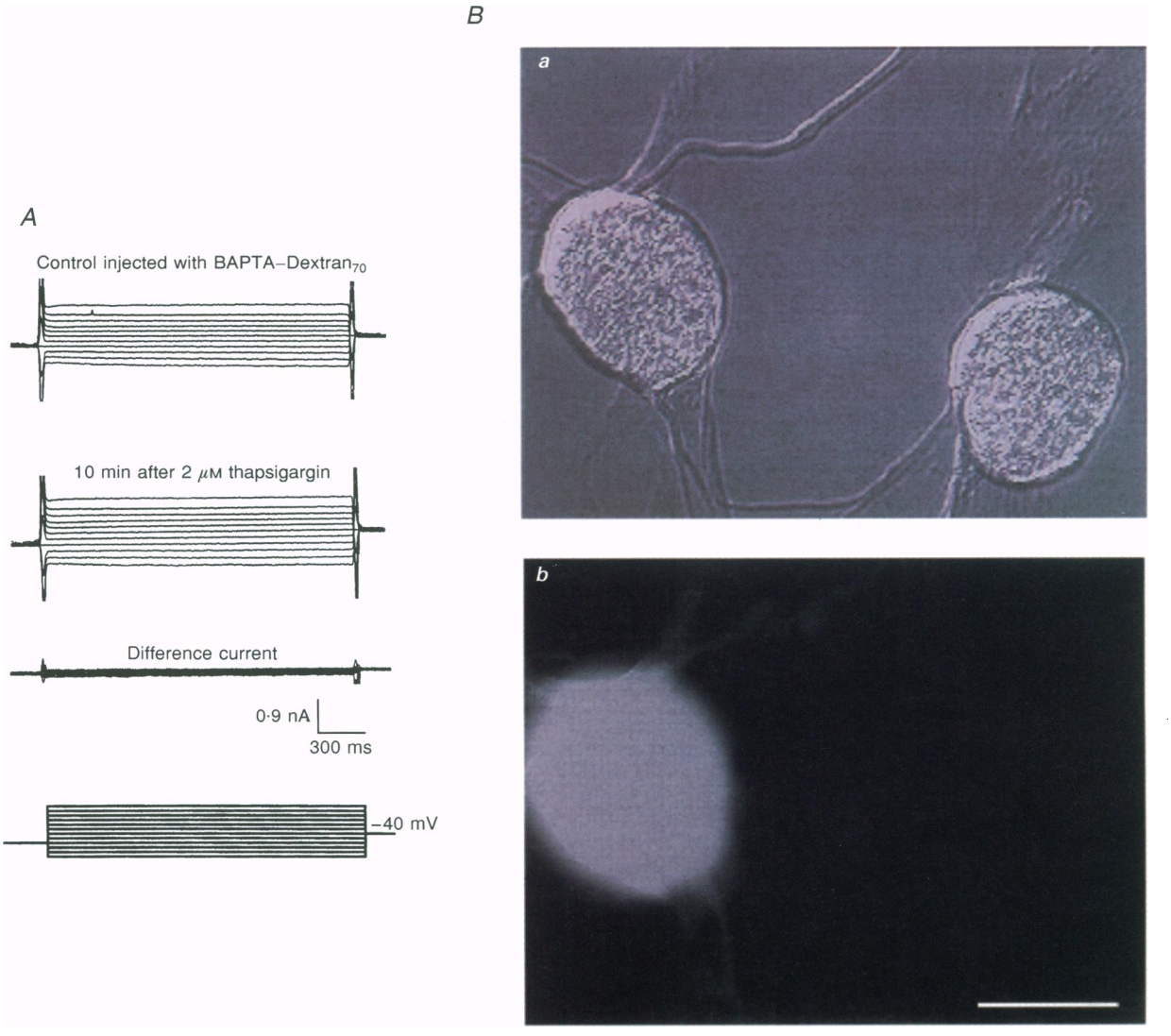

Figure 5. Effect of BAPTA–Dextran$_{70}$ on I_{Tg}

A, microinjection of the Ca^{2+} chelator, BAPTA–Dextran$_{70}$, inhibited the activation of I_{Tg}. Lucifer Yellow was included in the injection so that successful injections could be determined by observing intracellular fluorescence. *B*, Nomarski (*a*) and fluorescence images (*b*) of a field containing 2 cells. The cell on the left was microinjected with BAPTA–Dextran$_{70}$ and a small amount of Lucifer Yellow. Scale bar, 45 μm.

current. We estimated the relative selectivity of I_{Tg} from reversal potential measurements made in ion-substituted ASWs. Figure 6 shows I–V relations for I_{Tg} recorded in ASWs containing different $[Na^+]_o$ and $[K^+]_o$. The mean reversal potential of I_{Tg} in normal ASW was $-19{\cdot}2 \pm 0{\cdot}94$ mV ($n = 8$). Data from a single experiment are shown by filled circles in Fig. 6. When the driving force for K^+ was decreased by increasing the extracellular $[K^+]$ from 10·4 to 50 mM, the reversal potential of I_{Tg} was shifted to more positive potentials ($19{\cdot}7 \pm 1{\cdot}3$ mV, $n = 8$) (Fig. 6). When the $[Na^+]_o$ was lowered to 46 mM, there was a left shift in the I–V relation of I_{Tg}, as shown by the squares in Fig. 6. The mean reversal potential of I_{Tg} under these conditions was $-29{\cdot}3 \pm 1{\cdot}8$ mV ($n = 8$). These results demonstrate that both Na^+ and K^+ contribute to I_{Tg}. Overall the results suggest that Ca^{2+} released from internal stores directly stimulates a depolarizing non-selective cation conductance in somata of bag cell neurons. This may be important for maintaining depolarization of bag cell clusters in the abdominal ganglion. For example, activation of I_{Tg} could facilitate the propagation of action potentials throughout the abdominal ganglion network during an after-discharge (Kaczmarek *et al.* 1978).

We also tested for effects of thapsigargin on voltage-activated Ca^{2+} currents (Ba^{2+} was used as charge carrier to increase the size of the inward current) and a K^+ current. In most cells no apparent effect could be detected on inward currents (6 of 8 cells). The small effect on inactivation of Ba^{2+} current shown in Fig. 7*A* was, however, observed in two of eight neurons, and may reflect Ca^{2+}-induced inactivation of Ca^{2+} current. The I–V plot for one of these experiments is shown in Fig. 7*A* (right). Thapsigargin also produced only minor effects on K^+ currents. Figure 7*B* shows an experiment in which I_{Tg} was induced in Ca^{2+}-free (0·5 mM EGTA) ASW in which Tris replaced Na^+, and shows that thapsigargin produced a small increase in a component of K^+ current, presumably due to intracellular Ca^{2+} release acting on Ca^{2+}-dependent K^+ currents.

Lack of effect of thapsigargin on the after-discharge

In the intact abdominal ganglion of *Aplysia*, brief stimulation of the pleuroabdominal connective nerve produces a depolarization of the neurites of bag cell neurons (Kaczmarek *et al.* 1978). As a result, an after-discharge lasting approximately 30 min is generated within the neurites, and during this after-discharge action potentials propagate towards the somata. To test if Ca^{2+} release from intracellular stores induced by thapsigargin can influence the characteristics of a normal after-discharge we stimulated discharges in isolated abdominal ganglia in the presence or absence of thapsigargin. Figure 8*A* shows the typical firing pattern of a bag cell after-discharge in response to a brief electrical stimulus applied to the pleuroabdominal connective nerve. Action potentials were recorded using an extracellular suction electrode (Loechner *et al.* 1992). Thapsigargin had no effect on baseline electrical activity before application of the electrical stimulus. Figure 8*B* shows an electrically evoked after-discharge recorded from the ganglion 50 min after the addition of thapsigargin (50 μM), and as shown by comparison with the unstimulated preparation in Fig. 8*A*, thapsigargin did not alter the firing frequency or duration of the electrically evoked after-discharge in any obvious way. The mean

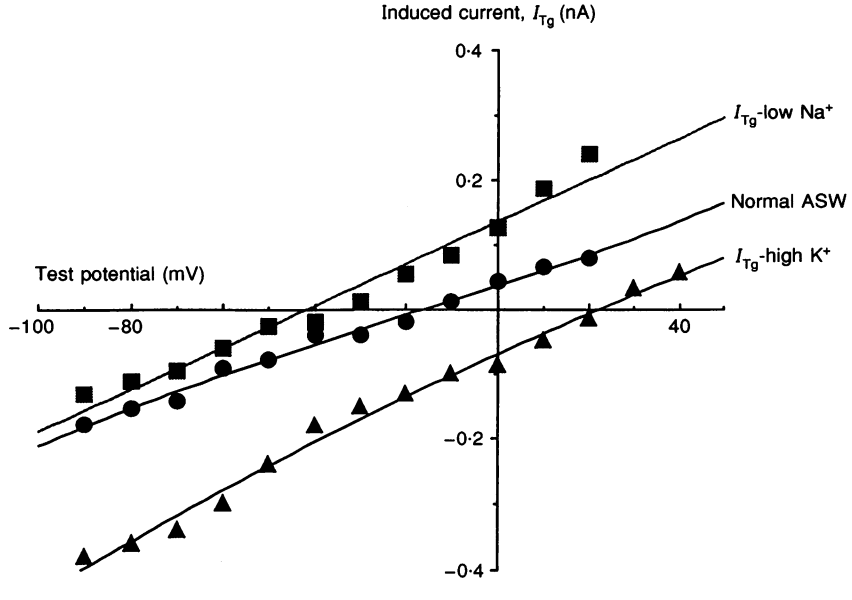

Figure 6. Reversal-potential measurements demonstrate I_{Tg} is a non-selective cation current

Mean I–V relations for I_{Tg} obtained in isosmotic substituted ASW in which the driving forces for K^+ (■) and Na^+ (▲) were respectively increased and decreased. The shifts in the reversal potentials observed under these conditions relative to the reversal potentials measured in normal ASW (●) demonstrate that both Na^+ and K^+ contribute to I_{Tg}.

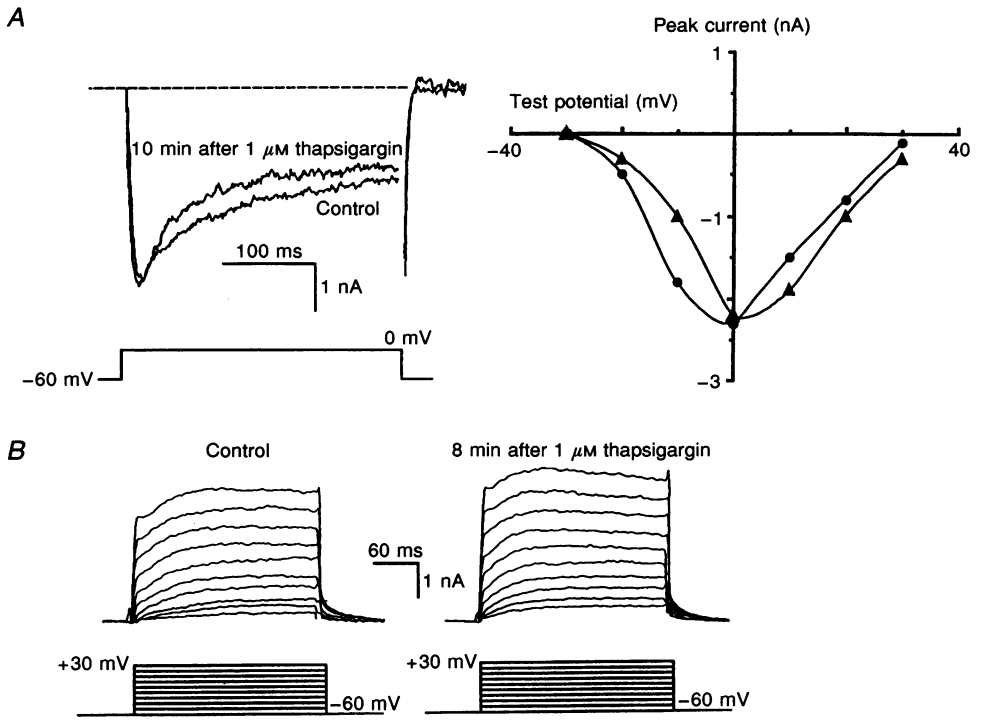

Figure 7. Effects of thapsigargin on voltage-activated Ca^{2+} and K^+ currents

A, voltage-gated Ba^{2+} currents before and 10 min after treatment with thapsigargin and an *I–V* plot for this type of experiment; ▲, control and ●, 10 min after 1 μM thapsigargin. Ba^{2+} was used as the charge carrier to increase the size of the inward current. *B*, voltage-dependent outward K^+ currents recorded in Ca^{2+}-free ASW before and after 1 μM thapsigargin.

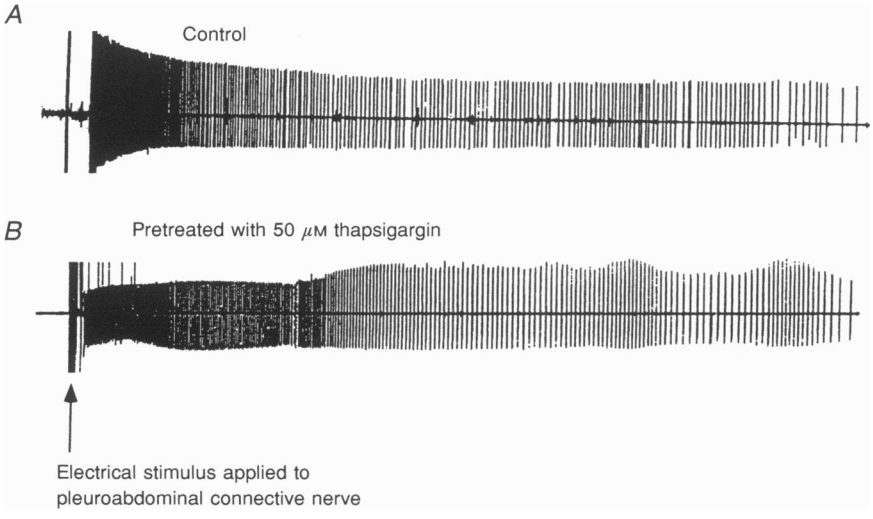

Figure 8. Lack of effect of thapsigargin on after-discharge

A, control bag cell after-discharge that lasted for 22 min. *B*, bath-applied thapsigargin (50 μM) did not alter in any way an after-discharge that was electrically stimulated 40 min after application of thapsigargin. Control after-discharges and those stimulated in the presence of thapsigargin had mean durations of 35·2 ± 1·8 min ($n = 4$) and 36·8 ± 3·1 min ($n = 4$), respectively.

durations of after-discharges in control and thapsigargin (50 μM)-treated preparations were $35{\cdot}2 \pm 1{\cdot}8$ min and $36{\cdot}8 \pm 3{\cdot}1$ min, respectively ($n = 4$ for each group). Similar results were obtained using 5 μM thapsigargin ($n = 4$).

DISCUSSION

Digital imaging of intracellular fura-2 fluorescence and the use of Ca^{2+}-selective vibrating microelectrodes on bag cell neurons has allowed us to measure spatial and temporal properties of intracellular Ca^{2+} release, and its associated plasma membrane Ca^{2+} flux. We have found that thapsigargin-sensitive Ca^{2+} stores are present in the bag cell neurons and that release from these Ca^{2+} stores activates a depolarizing non-selective cation conductance. We also demonstrated that release of intracellular Ca^{2+} stimulates Ca^{2+} influx across the plasma membrane, which is followed by extrusion of Ca^{2+} across the plasma membrane.

Initiation of the after-discharge occurs in bag cell neurites and intracellular Ca^{2+} release has been shown to occur during the after-discharge (Fisher *et al.* 1994). Our results with thapsigargin in bag cell neurons in intact abdominal ganglia suggest that Ca^{2+} release from thapsigargin-sensitive sites is not likely to be responsible for triggering normal after-discharges. It is possible, however, that the depolarization induced by Ca^{2+} release contributes to the normal maintenance of the after-discharge and to the ability of action potentials to propagate from the neurites, where the discharge is initiated, towards the somata. Maintained depolarization of somata may facilitate action potential propagation from bag cell neurons to target neurons throughout the *Aplysia* nervous system.

Effect of intracellular Ca^{2+} release on plasma membrane Ca^{2+} flux

Our observations suggest that a significant component of intracellular Ca^{2+} that accumulates during thapsigargin treatment is exported from the cells by a mechanism dependent on extracellular Na^+, which differs from a recently described Na^+-independent regulation of intracellular Ca^{2+} in voltage-clamped snail neurones (Kennedy & Thomas, 1995). Use of the Ca^{2+}-selective vibrating-probe technique allowed us to compare steady-state plasma membrane Ca^{2+} gradients in control and thapsigargin-stimulated neurons. The recordings revealed that intracellular Ca^{2+} release was followed by a sustained reduction of Ca^{2+} efflux, the time course of which correlates with the increase in cytoplasmic Ca^{2+} levels observed in fura-2-loaded neurons. A likely explanation for the Ca^{2+} influx is that thapsigargin induces Ca^{2+} entry across the plasma membrane. This Ca^{2+} influx may be related to Ca^{2+} depletion-activated currents, such as I_{CRAC} (Ca^{2+} release-activated current) or I_{DAC}(depletion-activated current), that may underlie capacitative Ca^{2+} entry in a variety of inexcitable cell types (Putney, 1990; Luckhoff & Clapham, 1994). Since thapsigargin is the most reliable stimulant of Ca^{2+} depletion-activated Ca^{2+} entry, our data suggest that an analogous Ca^{2+}-repletion mechanism may be present in bag cell neurons. To date, a second messenger that regulates capacitive Ca^{2+} entry has not been identified, although Randriamampita & Tsien (1993) have partially characterized a Ca^{2+} influx factor substance (CIF) that is generated intracellularly when intracellular Ca^{2+} stores are depleted by thapsigargin.

Neurites *versus* somata: spatial and temporal properties of thapsigargin-sensitive Ca^{2+} release

Following treatment with thapsigargin, the elevation of Ca^{2+} reached in neurites of bag cell neurons is less than that in somata. There may be several reasons for this. For example, the amount of thapsigargin-sensitive Ca^{2+} stores may be less in neurites. Alternatively, because neurites have a smaller volume than somata, Ca^{2+} pumps or Na^+–Ca^{2+} exchangers (Levy & Tillotson, 1988) may extrude Ca^{2+} from neurites at a rate that exceeds Ca^{2+}-extrusion efficiency in somata, or there may be a higher density of Ca^{2+}-sequestering proteins in neurites relative to somata. It is also likely that the mechanisms that regulate Ca^{2+} levels in bag cell neurons following action potential-driven Ca^{2+} influx differ from those in rat neocortical neurons, since Ca^{2+} transients in these neurons are prolonged by blockers of endoplasmic reticulum Ca^{2+}-ATPase, thapsigargin and cyclopiazonic acid (Markram, Helm & Sakmann, 1995), whereas these drugs do not alter the decay time constant of action potential-induced transients in bag cell neurons (R. J. Knox, E. A. Jonas, L. K. Kaczmarek & J. A. Connor, unpublished observations).

Possible physiological functions of cation current

Our results have demonstrated that intracellular Ca^{2+} release by thapsigargin produces a depolarization of bag cell neurons, probably because of the activation of a voltage-independent non-selective cation current I_{Tg}. This current could be recorded in extracellular solutions containing Na^+, K^+, TEA and Tris, but not in NMDG-substituted ASW or in neurons injected with the intracellular Ca^{2+} chelator, BAPTA–$Dextran_{70}$.

Non-selective cation currents were first described in heart (Kass, Lederer, Tsien & Weingart, 1978), where they have been implicated in pacemaker activity, and may also be regulated by Ca^{2+} release from intracellular stores (Colquhoun, Neher, Reuter & Stevens, 1981; Reuter, 1984). Similar currents are thought to account for the bursting behaviour of *Helix* neurons, where they provide a maintained depolarizing current that is critical to pacemaking activity (Partridge & Swandulla, 1987). Other functions have also been proposed for Ca^{2+}-activated non-selective cation currents (Bevan, Gray & Ritchie, 1984; Lee, Dayanithi, Nordmann & Lemos, 1992). In some non-excitable cells, non-selective cation channels that are Ca^{2+}

permeable are thought to provide one type of mechanism for capacitative Ca^{2+} entry following depletion of intracellular stores. For example, in mast cells, Ca^{2+} influx can occur by two independent mechanisms: a Ca^{2+}-selective current (I_{CRAC}) that is activated by depletion of internal Ca^{2+} stores (Hoth & Penner, 1993), and by non-selective cation channels that are permeable to other divalent cations (Penner, Matthews & Neher, 1988). The latter channels also contribute to the plateau phase of elevated $[Ca^{2+}]_i$ following receptor-operated release of Ca^{2+} from internal stores. Following stimulation of the bag cell neurons, there is an elevation of intracellular calcium that can be attributed to release from intracellular stores (Fisher *et al.* 1994). The cation current, I_{Tg}, that is thereby activated may contribute to the depolarization that is maintained during the afterdischarge, and may conceivably also contribute to the refilling of calcium stores.

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